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Direct Sequencing Disulfide-linked Peptide with Electrospray Ionization Tandem Mass Spectrometry

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Dissociation of disulfide is normally mandatory prior to disulfide peptide sequencing via electrospray ionization collision induced dissociation mass spectrometry (ESI-CID-MS). Herein, a facile method for directly sequencing intact disulfide peptide was proposed. Basic principles involved were electrolyte-enhanced corona discharge in ESI and the following oxidative cleavage reaction.

Mass spectrometry (MS) has aroused broad interest for its distinct ability of mass and charge characterization, and its accessibility of structural information of large biomolecules.¹ In the realm of mass spectrometry, electrospray ionization (ESI)² is one of the most widely-used atmospheric ion sources for quantitative and qualitative analysis of peptide and protein.³ In order to extend the utility of ESI, a series of atmospheric ion sources have been developed based on electrospray, such as nanoelectrospray ionization,⁴ desorption electrospray ionization (DESI),⁵ geometry-independent-DESI,⁶ electrostatic spray ionization,⁷ extractive electrospray ionization,⁸ induced electrospray ionization (iESI)⁹ and others.¹⁰

With the increasing development of these ionization techniques, MS-based protocols have drawn more and more attention for protein analysis,¹¹ such as *de novo* sequencing.¹² However, in traditional MS protocol for *de novo* sequencing, cleavage of disulfide is mandatory for disulfide-linked proteins prior to conventional collision-induced-dissociation (CID) MS analysis.^{13, 14} Many attempts, including chemical reduction¹⁵ and electrochemical reduction,¹⁶ have been increasingly reported to solve this issue. Nevertheless, methods based on effective but tedious wet-chemistry are frequently sample- and time-consuming, which would limit its application in analysis of real sample with small amount. To simplify the pretreatment process, MS-based online disulfide cleavage protocols are increasingly emerging, such as in-source reduction,¹⁷ thermal dissociation,¹⁸ electron capture dissociation (ECD),¹⁹ and electron transfer dissociation (ETD).²⁰ Ultraviolet (UV) was also employed for disulfide dissociation during ionization^{21, 22} or inside mass analyzer²³. Low temperature plasma (LTP) was also used by Xia et al. to generate reactive oxygen species for radical-attacked disulfide cleavage during ESI.¹³ Recently, we also used corona discharge (CD) in iESI¹⁴ to dissociate disulfide bond for increased peptide sequence coverage. In these studies (UV, LTP and iESI), oxidative

cleavage reaction (OCR) between hydroxyl radical (HO•) and disulfide was demonstrated to be the mechanism of disulfide dissociation.^{13, 14, 22} Moreover, the reactive HO• in iESI was proved to be originated from CD, which was monitored with a photomultiplier tube (PMT). Though these methods were effective for disulfide cleavage, additional instrumental modifications (including UV lamp, additional devices and power supplies for LTP and iESI) were necessary for CD generation. Recently, CD in conventional ESI was reported to produce reactive oxygen species (such as HO•).²⁴ Although point-plane CD has been thoroughly investigated^{25, 26} and widely utilized for applied physics²⁵, spectroscopy^{27, 28} and scientific instruments,^{28, 29} systematic investigation of CD in ESI is still lacking.

In this study, we aimed to employ CD in conventional ESI for in-situ disulfide cleavage, with minimal instrumental modification on conventional ESI. Intact disulfide peptides/proteins, including selectin binding peptide, oxidized glutathione (GSSG) and human insulin, were successfully sequenced with the absence of pre-dissociation of disulfide bond. Therefore, a facile method of direct sequencing of intact disulfide peptide/protein was proposed based on conventional ESI-CID-MS with electrolyte-enhanced CD in ESI and the following OCR.

Electrospray-based light (ESL) emission, a known sign of CD,²⁴ was defined to quantitatively characterize CD during ESI processes. A PMT was placed under the ESI spray emitter in a dark room (**Fig. 1a**). With this setup, influencing factors of electrolyte species, spray voltage, flow rate, emitter size, and distance between emitter and counter-electrode on CD were investigated. After optimization of these operating parameters, in situ disulfide cleavage could be achieved with conventional ESI setup (**Fig. 1c**).

Step voltage was applied in a time-dependent manner and corresponding ESL signal was monitored in real time. **Fig. 1b** showed the step voltage-ESL curves regarding the effect of electrolyte species and other parameters were presented in **Fig. S1**. With the same electrolyte concentration (10 mM), we found that ESL signal kept background level regardless of the electrolyte species before the spray was initiated. Moreover, the ESL intensity depended heavily on the electrolyte species and spray voltage once the spray was initiated. Electrolyte with high valence state of cations (such as FeCl₃) was observed with stronger CD than the one with low valence state (such as NaCl). (**Fig. 1b & S1a**) This result may

be due to the higher electrical conductivity of electrolyte with higher valence state so that CD could be initiated more easily. Exceptionally, NH_4OAc generated relatively low ESL intensity than NaCl under higher spray voltage though the cations and anions of these two electrolytes are of the same valence state. This difference might be due to the diversity in volatility between NH_4OAc and NaCl , in which NH_4OAc is more easily volatilized and decomposed, facilitating the generation of CD. Furthermore, flow rate influenced the ESL regularly. Low flow rate (0.1–1 $\mu\text{L}/\text{min}$) would facilitate the occurrence of CD. (Fig. S1b) CD was also found to be sensitive to the distance between emitter and counter-electrode, as well as the spray emitter size. Increasing the distance would reduce the degree of CD (Fig. S1c) and increasing the emitter size would elevate the degree of CD (Fig. S1d). Therefore, quantitative and ocular evaluation of CD could be obtained via this step voltage-ESL method.

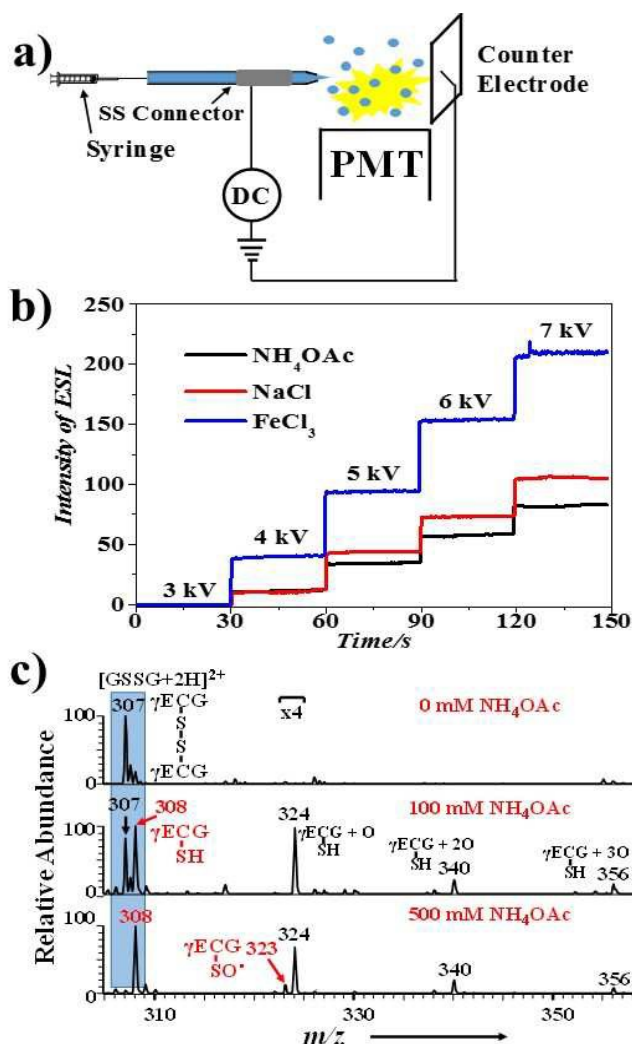


Fig. 1 a) Schematic representation of conventional ESI setup equipped with a photomultiplier tube (PMT) for measuring the intensity of electrospray-based light emission (ESL), a known sign of CD. Note that the scheme is not proportionally scaled. SS, stainless steel. b) Step voltage-ESL curves to study the effects of electrolyte on ESL response. With regard to the electrolyte, water solution were added with various equal molar of 10 mM electrolyte (NH_4OAc , NaCl , FeCl_3) respectively. c) The dependence of disulfide cleavage in GSSG (8 μM) on the concentration of NH_4OAc . (Full

MS spectra with a broader m/z range seen in Fig. S2.) With the increase of NH_4OAc in concentration, the cleaved product (GSH) and the oxidized product (GSO^\bullet) get increased information in the MS spectrum.

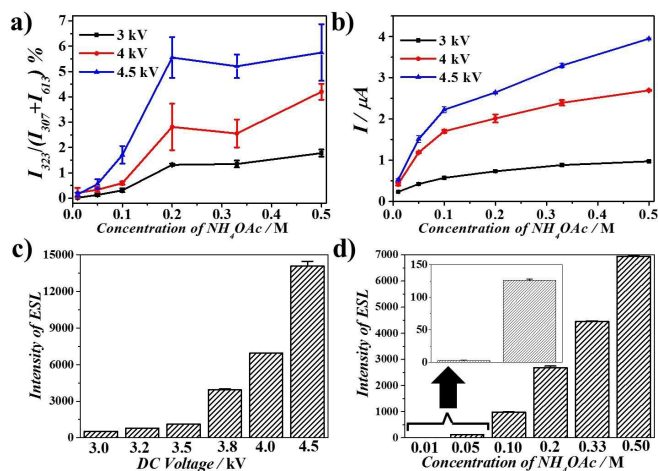


Fig. 2 Titration experiments for the dependence of OCR on the concentration of NH_4OAc . The dependence of cleavage of disulfide in GSSG on the concentration of NH_4OAc was explored under different spray voltage. The increase trend of GSO^\bullet , the characteristic cleavage product, was shown in a). The increase trend in spray current with varied NH_4OAc concentration was presented in b). The dependence of ESL intensity on spray voltage (keep constant NH_4OAc concentration of 0.1 M) and the concentration of NH_4OAc (keep constant DC voltage of 3 kV) were also depicted in c) and d), respectively.

As a practical application of electrolyte-enhanced CD in ESI, we then tried to utilize it for disulfide cleavage. Recently, the iESI-based disulfide cleavage has been demonstrated to be due to the OCR between disulfide and HO^\bullet , i.e. it is originated from enhanced CD.¹⁴ In this study, similar cleavage pathway was made for disulfide dissociation but without modifications of the conventional ESI power supply.

In order to explore the possibility of electrolyte-enhanced CD in ESI for disulfide cleavage, we performed a series of titration experiments (Fig. 1c & Fig. 2) for the dependence of OCR on the concentration of NH_4OAc . GSSG, with an interchain disulfide, was chosen as the model compound. Various amounts of electrolyte ranging from 10 mM to 500 mM were added into the spray solution (8 μM GSSG in water) for ESI analysis. Conventional ESI-MS spectrum of GSSG without electrolyte was observed to give no cleavage products. With the increase of NH_4OAc content, the cleavage product, GSH and GSO^\bullet , were observed with increased percentage in the MS¹ spectrum (Fig. 1c). GSH and GSO^\bullet are pair-products from disulfide dissociation, as shown in previous reports.¹³ Interestingly, the ratio of GSO^\bullet and GSSG were found to be increased until saturation with the elevation of the concentration of NH_4OAc (Fig. 2a). Similar trend was observed for GSH and GSSG. (Fig. S3) These observations indicated that disulfide of GSSG was under increasing dissociation when increasing the concentration of NH_4OAc . Similar OCR fragmentations were observed when NH_4HCO_3 was tested, and lower concentration of NH_4HCO_3 is needed than NH_4OAc to obtain the same degree of OCR.

In line with the independent step voltage-ESL monitoring experiment, CD was observed to increase regularly with the increase of spray voltage (Fig. 2c) and NH_4OAc concentration (Fig. 2d).

Similar changing trend appeared in the spray current versus NH_4OAc concentration curves (Fig. 2b). Therefore, direct relationship between CD and disulfide cleavage in ESI was proved, in a way similar as that in iESI.¹⁴ The ratios of GSH/GSSG and GSO•/GSSG were also found to be increased with increased amount of GSSG (Fig. S4). Thus, electrolyte-enhanced CD in ESI was demonstrated to be able to dissociate disulfide for peptide sequence analysis.

Furthermore, based on electrolyte-enhanced CD in ESI and OCR between disulfide and $\text{HO}\cdot$, direct sequencing of intact disulfide-containing protein was exemplified with native human insulin. Fig. 3a/b showed the reactive MS spectrum of native insulin in the presence of various concentrations of electrolyte (NH_4OAc). Radical additive ion ($[\text{M} + 5\text{H} + \text{OH}]^{5+}$, m/z 1166) was observed to be positively correlated with electrolyte content (Table S1 & S5, S6), implying that disulfide in insulin was increasingly dissociated. Proposed cleavage pathways and corresponding identified MS/MS product ions were summarized in Scheme S1. Sequence coverage of native human insulin derived from CID (Fig. 3c & S5) of radical additive ion was 59% (12 cleavages beyond disulfide, 17 cleavages inside disulfide, and total possible 49 cleavages. Inset in Fig. 3c), which is comparable with our previously proposed iESI-based protocol (61%)¹⁴ and other non-CID techniques (such as ETD, 64%)³⁰. The present method using ESI-CID-MS was proved to be practical for direct sequencing of intact disulfide peptide/protein with minimal instrumental modification (only requiring addition of electrolytes in the spray solvent).

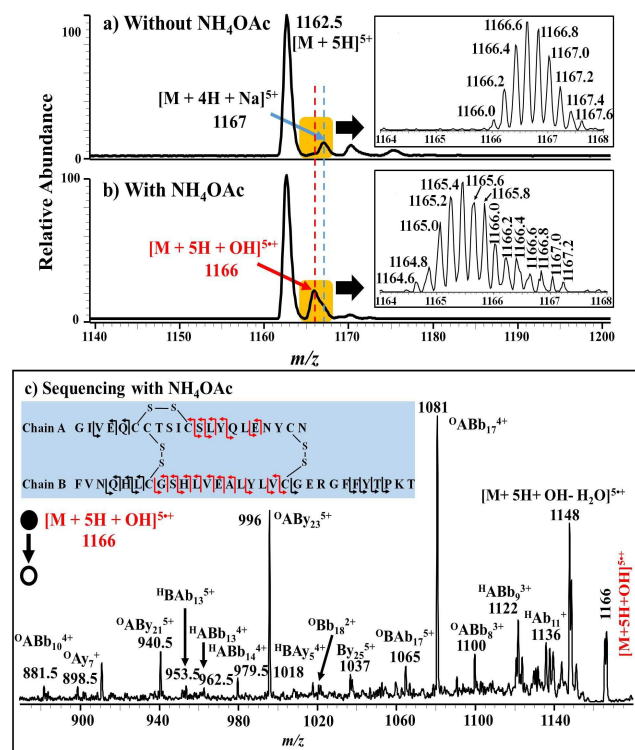


Fig. 3 Facile sequencing of human insulin with electrolyte-enhanced CD in conventional ESI. a) The significant increment in MS¹ spectrum of radical additive ion ($[\text{M} + 5\text{H} + \text{OH}]^{5+}$, m/z 1166) confirmed the oxidative cleavage pathway, which was further strengthened by its CID MS/MS characteristic fragments. In order to distinguish the radical additive ion from sodium adduct ion, zoom scan mode was introduced and resulting spectrum were inserted in a) and b). Major fragments derived from insulin with 100 mM

NH_4OAc were showed in c) and more details and control experimental results seen in supporting information (Fig. S5~7).

However, adding electrolytes in spray solvent would not be always suggested under certain circumstances, i.e. HPLC-ESI-MS, a routinely-used technique for biochemical analysis.³¹ Then to keep the LC separation efficiency of disulfide-containing peptide/protein, we also developed a dual channel ESI-CID-MS protocol (Fig. 4a). This dual channel ESI setup could remain all LC separation conditions (using traditional LC-ESI interface to replace **emitter 1**). As a proof-of-principle validation of direct sequencing native and intact disulfide-containing peptide, **emitter 1** was loaded with disulfide-containing peptide and **emitter 2** was loaded with concentrated electrolyte. When high voltage was turned on only for the **emitter 1** (single channel ESI, Fig. 4a), sequence information was not observed (Fig. 4b). When high voltage was turned on for both emitters (dual channel ESI, Fig. 4a), selectin binding peptide (${}_{\text{S}}\text{CIELLQARC}_{\text{S}}$) cleavage was typically identified (Fig. 4c & Fig. S8) and the sequence coverage was as high as 87.5% (Inset in Fig. 4c). Similar results could be obtained from the dual channel ESI-CID-MS analysis of GSSG (Fig. S9). Besides, in order to compare the performance of disulfide cleavage between dual channel and single channel ESI protocol, the disulfide in selectin binding peptide was subjected to dissociation via single channel ESI, in which concentrated electrolyte and disulfide peptide were co-sprayed from single spray emitter. Results from reaction spectra and tandem MS spectrum (Fig. S10, Fig. S8 & Fig. 4c) indicated that, dual channel ESI protocol was more advantageous than single channel ESI for disulfide cleavage in terms of analyte intensity and reaction purity, though decrease of reaction efficiency was observed for dual channel ESI. Thus, it could also be concluded that charge competition effect from concentrated electrolytes in single channel ESI could be alleviated by using the dual channel ESI-based protocol. In addition, the formation of protonated disulfide-containing peptide (**emitter 1**, for cleavage) and generation of $\text{HO}\cdot$ (**emitter 2**, for CD) using two individual ESI emitters further proved the CD-related mechanism of OCR between disulfide bond and $\text{HO}\cdot$.

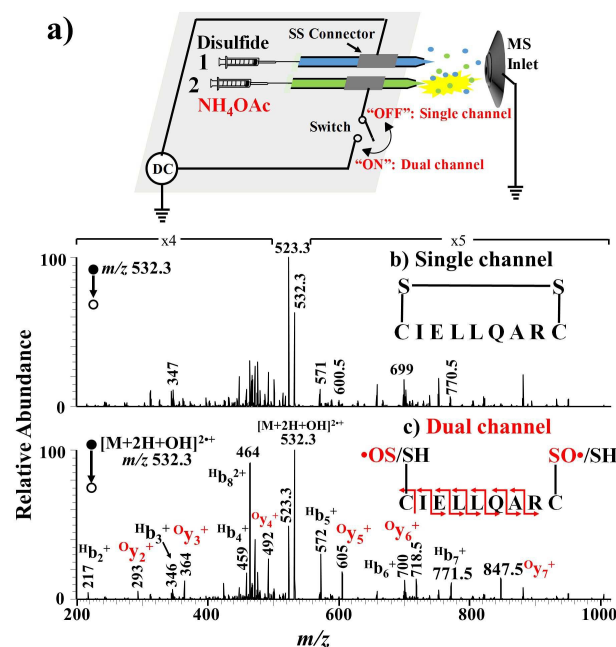


Fig. 4 Dual channel ESI-CID-MS for selectin binding peptide sequencing. a) Diagrammatic layout of experimental setup. Selectin

1 binding peptide (10 μ M) was single channel sprayed (switch “OFF”) or dual channel sprayed (switch “ON”). For dual channel, significantly increased sequence coverage (c) was obtained compared with conventional single channel ESI (b).

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6 In summary, direct sequencing of intact disulfide-containing peptide/protein using ESI-CID-MS was demonstrated with a new protocol based on electrolyte-enhanced CD in ESI and OCR between disulfide and HO \cdot . An ocular and quantitative method of step voltage-ESL monitor for CD characterization was also proposed. We also provided new evidences of the influencing factors of CD in ESI, a widely-used soft ion source of mass spectrometry for biological and chemical research. Dual channel ESI setup was further designed to extend the versatility of present ESI-based sequencing method. Though we now aimed to present an in situ and rapid sequencing method of native disulfide peptide/protein, sequencing denatured disulfide protein collected from HPLC would be potentially accomplished. This rapid, facile disulfide cleavage protocol with minimal instrumental modification would enable improving the performance of ESI-CID-MS for disulfide-containing peptide sequencing.

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Though the proved effectiveness of present method for disulfide-containing peptide sequencing, some limitations still exist, such as the observed low yield of OCR product. Presumably, this fact should be related with unexpected side reactions and stability of OCR product. The unexpected side reactions, such as electron-induced disulfide cleavage and sequentially oxidizing reaction, could be in some extent suppressed by precisely controlled addition of organic solvents. In addition, as a result of the intrinsic characteristic of weak stability of radical additive peptide ions, the significantly lower intensity of GSO \cdot than that of GSH and GSH+O is observed in **Fig. 1c**.

All experiments were carried out with a LTQ-Velos Pro (Thermo Fisher Scientific, CA, USA) mass spectrometer. The ESL signal was taken with a PMT supplied by Xianruimai Analytical Instruments Co., Ltd. (Changchun, China). More detailed instrumental parameters seen in supplementary information.

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Notes and references

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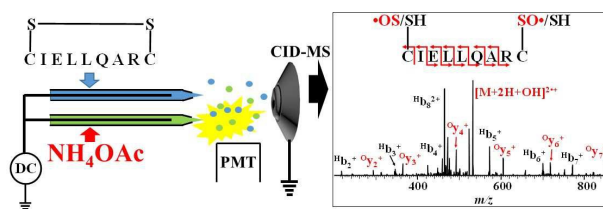
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6 **TOC graphic:**
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16 Enhanced corona discharge was employed for in-spray dissociation of disulfide bonds, facilitating
17 disulfide-containing peptide sequencing with ESI-MS/MS.
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